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Molecular and cellular pathology of very-long-chain acyl-CoA dehydrogenase deficiency

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ABSTRACT

Background: Very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiency (VLCADD) is diagnosed in the US through newborn screening (NBS). NBS often unequivocally identifies affected individuals, but a growing number of variant patterns can represent mild disease or heterozygous carriers.
Aims: To evaluate the validity of standard diagnostic procedures for VLCADD by using functional in vitro tools.
Methods: We retrospectively investigated 13 patient samples referred to our laboratory because of a suspicion of VLCADD but with some uncertainty to the diagnosis. All 13 patients were suspected of having VLCADD either because of abnormal NBS or suggestive clinical findings. *ACADVL* genomic DNA sequencing data were available for twelve of them. Ten of the patients had an abnormal NBS suggestive of VLCADD, with three samples showing equivocal results. Three exhibited suggestive clinical findings and blood acylcarnitine profile (two of them had a normal NBS and the third one was unscreened). Assay of VLCAD activity and immunoblotting or immunohistologic staining for VLCAD were performed on fibroblasts. Prokaryotic mutagenesis and expression studies were performed for nine uncharacterized *ACADVL* missense mutations.
Results: VLCAD activity was abnormal in fibroblast cells from 9 patients (8 identified through abnormal NBS, 1 through clinical symptoms). For these 9 patients, immunoblotting/staining showed the variable presence of VLCAD; all but one had two mutated alleles. Two patients with equivocal NBS results (and a heterozygous genotype) and the two patients with normal NBS exhibited normal VLCAD activity and normal VLCAD protein on immunoblotting/staining thus ruling out VLCAD deficiency. Nine pathogenic missense mutations were characterized with prokaryotic expression studies and showed a decrease in enzyme activity and variable stability of VLCAD antigen.
Conclusions: These results emphasize the importance of functional investigation of abnormal NBS or clinical testing suggestive but not diagnostic of VLCADD. A larger prospective study is necessary to better define the clinical and metabolic ramifications of the defects identified in such patients.

1. Introduction

Very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiency (VLCADD) is a disorder of long chain mitochondrial fatty acid oxidation (FAO) [1,2] with more than 400 reported patients [3]. VLCADD can present with a variety of clinical symptoms and a spectrum of severity that ranges from severe life threatening illness in the newborn period to relatively mild disease first developing late in childhood or early adulthood. Two major childhood phenotypes have been recognized [4–6]. The first consists of severe neonatal or early onset disease with recurrent episodes of hypoglycemia, acidosis, hepatic dysfunction, and cardiomyopathy. Patients who survive their initial presentation exhibit progressive cardiomyopathy and have a reported 75% mortality rate in the first few years of life. Children with later onset symptoms can have repeated episodes of hypoketotic hypoglycemia but are at low risk of developing cardiomyopathy, with a resultant lower mortality and better long term prognosis. Sequence analysis of the *ACADVL* gene has revealed some correlation of mutation genotype with phenotype however, this relationship is imperfect [5,7]. Not surprisingly, patients with null mutations leading to complete absence of VLCAD tend to have more severe symptoms than those with some residual enzyme activity.

VLCADD is readily identified by newborn screening (NBS) of acylcarnitine profiles from blood spots with tandem mass spectrometry (often referred to as expanded newborn screening) in numerous countries, including the US, and has emerged as the second most common inborn error of fatty acid oxidation [6,8–12]. The natural

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history of VLCADD has been radically modified by NBS due to pre-symptomatic treatment, with great improvement in short-term outcome [6,13–15]. Implementation of such treatment requires that follow-up procedures for confirmatory diagnosis for individuals flagged by NBS be as accurate as possible.

The acyl-CoA dehydrogenases (ACADs) are a family of enzymes that catalyze the α,β-dehydrogenation of acyl-CoA esters, transferring electrons to electron transferring flavoprotein (ETF) [16]. At least nine members of this enzyme family have been identified, each with a characteristic substrate specificity profile [17–23]. Very long, medium and short chain acyl-CoA dehydrogenases (VLCAD, MCAD, and SCAD respectively) catalyze the first step in the β-oxidation cycle with substrate optima of 16, 8 and 4 carbon chains respectively. ACAD9 is active against both saturated and unsaturated long chain substrates [24,25]. The physiologic role of long chain acyl-CoA dehydrogenase (LCAD) remains unknown [26]. All ACADs are encoded in the nuclear genome as a precursor protein and function in the mitochondria [27–29]. While mature VLCAD and ACAD9 are homodimers, the other ACADs are homotetramers with each monomer containing a non-covalently bound flavin adenine dinucleotide molecule (FAD) as a prosthetic group [17,30,31]. VLCAD protein shares homology with other ACADs over much of its length, but has an additional 180 amino acids at its carboxy terminus which facilitates its interaction with the inner mitochondrial membrane and other fatty acid oxidation proteins [32–34].

In an attempt to evaluate the validity of the diagnostic procedures clinically available for VLCADD (i.e. blood acylcarnitine profile and molecular genetic testing of *ACADVL*), we used a combination of functional tests to retrospectively characterize enzymatic, biochemical, and molecular data of suspected VLCADD patients referred to our laboratory in the last three years with special emphasis on samples with some uncertainty raised by initial test results.

2. Materials and methods

2.1. Fibroblast studies

2.1.1. Cell culture

Patient skin biopsies for fibroblast culture were performed on a clinical basis with written informed consent from patients and/or parents. Fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 100 μg/ml penicillin/streptomycin, 4.5 g/l glucose, 4 mM glutamine and 2 mM pyruvate. At 90% confluence in a T175 flask, cells were harvested by trypsinization, pelleted and stored at −80 °C for further studies.

2.1.2. Western blot

Cell pellets were resuspended in 250 μl ice-cold water and subjected to sonication on ice and centrifuged at 18,000 ×*g* for 20 min at +4 °C to remove cell debris. The supernatant was used for Western blotting as described [33]. Briefly, 50 μg protein of an SDS treated sample was loaded onto the gel. Following electrophoresis, the gel was blotted onto a nitrocellulose membrane followed by visualization with a primary rabbit anti-VLCAD polyclonal antibody and secondary alkaline phosphatase goat anti-rabbit IgG antibody. Anti-VLCAD serum was produced as already reported [33].

2.1.3. Confocal imaging of fibroblasts

Fibroblasts from patients and healthy controls were seeded at a concentration of 5×10^4 cells/ml on tissue culture-treated glass cover slips and allowed to grow overnight at 37 °C in a 5% CO_2, 95% humidity incubator. Cells were then fixed in 4% paraformaldehyde for 10 min followed by 0.1% Triton X100 cell permeabilization and further blocked after brief washings in 5% donkey serum (Jackson ImmunoResearch, West Grove, PA) for 1 h on ice. This was followed by double primary antibody incubation with 1) anti-VLCAD antibody and 2) anti cytochrome *c* oxidase subunit 4 antibody (Abcam, Cambridge, MA) at 4 °C overnight. After brief washing, cells were further incubated with donkey anti-rabbit secondary antibody Alexa Fluor 488 (Invitrogen, Grand Island, NY) for VLCAD, and donkey anti-mouse secondary antibody Alexa Fluor 555 (Invitrogen) for cytochrome *c* oxidase subunit 4. Nuclei were counterstained with DAPI. The cover slips were then mounted using mounting media before imaging. All the images were taken using an Olympus Confocal FluoView FV1000 microscope at a magnification of 60×.

2.1.4. VLCAD activity measurement

Fibroblast pellets were resuspended in 250 μl 50 mM Tris–Cl, pH 8.0, 10 mM EDTA, subjected to sonication on ice and centrifuged at 18,000 ×*g* for 20 min at 4 °C. The supernatant was assayed for VLCAD activity with the highly sensitive and specific electron transfer flavoprotein fluorescence reduction assay as described using 100 to 150 μg protein [35,36].

2.2. Mutagenesis and expression studies

For prokaryotic mutagenesis studies, nine mutations (R162H, I189T, G289R, I420L, G439D, M443R, G514E, L540P and R567Q) were introduced into the VLCAD ΔEx3 pET-21a(+) expression construct using the QuickChange Site-Directed Mutagenesis Kit according to the manufacturer's instructions (Stratagene, La Jolla, CA). Mutations were verified by sequencing and the plasmids were introduced into an *Escherichia coli* expression strain (BL21), cultured at 37 °C and induced for expression studies as previously described [33]. Enzyme activity and Western blotting were performed on cell-free extracts as above.

3. Results

3.1. Patients and mutations

Thirteen patients with a suspicion of VLCADD were retrospectively studied (Table 1). Seven patients (PTs 1, 3, 4, 5, 6, 9 and 10) had been flagged by newborn screening as unequivocally suggestive of VLCADD due to a clearly elevated C14:1 and/or C14 acylcarnitine levels. For 3 patients (PTs 8, 12 and 13), the results of the NBS were equivocal for VLCADD (Table 1). For the other patients, NBS was normal in two (PTs 2 and 7) and not performed in one (PT 11), but all three exhibited clinical symptoms considered suggestive of VLCADD (Table 1). Blood acylcarnitine profiling was diagnostic of VLCADD in PT 11 but only showed non-specific abnormalities in PTs 2 and 7. *ACADVL* genomic DNA sequencing data are shown in Table 1. Nine patients had two identified mutations, while three had only one mutated allele (PTs 8, 12, 13). Six new missense mutations (R162H, I189T, I420L, M443R, G514E, L540P), one previously reported [5,37,38] and functionally characterized [33] mutation (V283A), and 4 reported but uncharacterized missense mutations (L202P, G289R, G439D, R567Q) [38–40] were identified. Two splice donor site mutations were identified. The first mutation, previously reported, (c.1182+1G>A) induces exon 11 skipping [41], while the second (c.1182+3G>T) is new. Two small indels, insertion of 9 base pairs (c.1707_1716dup) and a 3 base-pair deletion (c.896_898del) are novel. The latter change induces a p.K299 deletion. This amino acid deletion has previously been reported to be caused by a different mutation (c.895_897del) [42]. Patient 11, whose mutations have previously been reported [43], harbored 2 known mutations creating a frame shift: a deletion of 2 base pairs (c887_888del) [44] on one allele and a splice mutation on the other (c.1679−6G>A) [45]. Patient 2 harbored two variants likely to be nonpathogenic (G43D, reported as a likely polymorphism [5], and L17F, reported as a nonpathogenic variant: http://www.ncbi.nlm.nih.gov/sites/varvu?gene=37). For the patients with only one *ACADVL* exonic mutation, a macro or micro gene deletion/duplication was ruled out by prior clinical microarray analysis.